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Adipose tissue-derived stromal cells acquire endothelial-like features upon reprogramming with SOX18

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Abstract Adipose tissue-derived stromal cells (ASC) form a rich source of autologous cells for use in regenerative medicine. *In vitro* induction of an endothelial phenotype may improve performance of ASCs in cardiovascular repair. Here, we report on an *in vitro* strategy using direct reprogramming of ASCs by means of ectopic expression of the endothelial-specific transcription factor SRY (sex determining region Y)-box18 (SOX18). SOX18 induces ASCs to express a set of genes involved in vascular patterning: MMP7, KDR, EFNB2, SEMA3G and CXCR4. Accordingly, SOX18 transduced ASCs reorganize under conditions of shear stress, display VEGF-induced chemotaxis and form tubular structures in 3D matrices in an MMP7-dependent manner. These *in vitro* findings provide insight into molecular and cellular processes downstream of SOX18 and show that reprogramming using SOX18 is sufficient to induce several endothelial-like features in ASCs.

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Introduction

Adipose tissue-derived stromal cells (ASCs) have been reported to diverge under specific culture conditions towards adipogenic, chondrogenic, myogenic and osteogenic cells (Zuk et al., 2001). This apparent plasticity, combined with the fact that these cells can be isolated in high numbers by minimally invasive methods, raised great interest in ASCs as a source of autologous cells for use in regenerative medicine. The use of ASCs in cardiovascular repair has been the subject of several *in vitro*- and animal studies and has moved towards clinical applications. Administration of adipose-derived cells, either freshly isolated or after conditioning in cell culture, was shown to improve left ventricular function in models of acute and chronic myocardial infarction. Likewise, in animal models of hind limb ischemia, favorable effects on revascularization have been reported (reviewed by Madonna et al. (2009)). The mechanisms through which ASCs exert these effects are not fully elucidated. ASCs have been reported to differentiate both *in vitro* and *in vivo* towards cardiomyocytic or endothelial phenotypes. In animal models it was observed that at least a part of the administered ASCs actually displays cardiomyocytic or endothelial markers and becomes structurally incorporated in the myocardium or vascular structures, respectively (Miranville et al., 2004; Planat-Benard et al., 2004; Yamada et al., 2006; Miyahara et al., 2006). In addition, it was shown that ASCs can function as a paracrine source of growth factors, cytokines and signaling molecules (Miyahara et al., 2006; Kondo et al., 2009). Thus, the view has emerged that ASCs support cardiovascular repair by providing local signals for cell recruitment and tissue survival as well as by physically substituting, in an adaptive manner, for lost cells.

Several papers describe attempts to specifically differentiate ASCs towards an endothelial phenotype (Miranville et al., 2004; Planat-Benard et al., 2004; Wosnitza et al., 2007; Kang et al., 2010; Konno et al., 2010). *In vitro* culture of ASCs in media supplemented with specific growth factors, in particular VEGF and bFGF, resulted in the expression of a limited range of endothelial markers, mostly with incomplete penetrance. In culture, cells did not display the typical endothelial morphology, nor did they organize in endothelial sheet-like structures. Therefore, the concept of conditioning ASCs to cells with an endothelial phenotype by stimulation with growth factors has proven partially successful but seems to require further optimization.

Sry-related high-mobility-group box (SOX) transcription factors are involved in maintaining stemness, differentiation and lineage commitment. Development of the vasculature is critically dependent on the SOXF subfamily, consisting of SOX7, SOX17 and SOX18 (Lefebvre et al., 2007; François et al., 2010). During mouse embryonic development, SOX18 is expressed in the developing cardiovascular system: in the allantois and yolk sac blood islands, in the developing heart and in the paired dorsal aorta. Expression is continued at sites of embryonal angiogenic- and vasculogenic expansion and was reported to re-occur in adult skin wounding (Pennisi et al., 2000a; Darby et al., 2001). In animal models, SOX7, SOX17 and SOX18 were found to have essential, partly redundant, roles in the formation of the vasculature during embryonic development (Pennisi et al., 2000b; François et al., 2008; Sakamoto et al., 2007; Herpers et al., 2008; Pendeville et al., 2008; Cermenati et al., 2008; Matsui et al., 2006; Sacilotto et al., 2013). However, their effects at the level of gene regulation and, thus, the molecular basis of their role in development are poorly understood. Applying overexpression and silencing of SOX18 in endothelial cells, our group has shown that SOX18 has a crucial role in both the transcription of the endothelial-specific tight junction protein claudin 5 and maintenance of the endothelial barrier (Fontijn et al., 2008). Recently, Hoeth et al. (2012) analyzed the transcriptome of endothelial cells overexpressing SOX18, pointing at a role for SOX18 in the morphogenesis of the vasculature. Using defined *in vitro* culture methods, two populations of angiogenic cells can be isolated from blood: early EPCs that contribute to vessel repair in a paracrine manner and endothelial colony forming cells (ECFCs), or outgrowth endothelial cells (OEC, late EPC), that have the capacity to physically form neo-vessels (reviewed in Favre et al., 2013). Using comparative genome-wide transcriptional profiling of these two cell populations, Medina et al. (2010) have shown that the mRNA fingerprint of ECFC closely resembles that of endothelial cells, and is accompanied by high, specific expression of SOX18. Thus, the available literature indicates a key role for SOX18 in vascular development and repair, in both embryonic and adult stages, and in specification of the endothelial phenotype. We therefore hypothesized that ectopic expression of SOX18 in ASCs might induce endothelial-like features and tested this reprogramming approach *in vitro*.

Materials and methods

Isolation of the stromal fraction of adipose tissue

Human subcutaneous adipose tissue samples from elective plastic surgery were obtained from the department of Plastic Surgery (Tergooi Hospital in Hilversum, The Netherlands) according to hospital guidelines after written informed consent. Adipose tissue was harvested from the abdomen after resection. In this study 7 female donors (age range: 40–61 years, mean: 47 years) were included. Procedures complied with the principles of the Declaration of Helsinki. After surgery, adipose tissue was stored in sterile phosphate-buffered saline (PBS) (B. Braun, Melsungen, Germany) at 4 °C overnight and processed within 24 h, as described previously (Naaijkens et al., 2010).

ASC culture and shear stress

ASCs were initially cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 5% platelet lysate, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) and 10 U/ml heparin (Leo Pharma, Amsterdam). In all experiments, early passages ASCs (p2–3) were used (Naaijkens et al., 2010).

Preceding SOX18 transduction, cells were cultured for 10 days on surfaces coated with collagen IV (Sigma Aldrich, St. Louis, MO, USA) in endothelial cell growth medium (EGM2) (Lonza, Walkersville, MD, USA) with a final concentration of 10 ng/ml (w/v) basic fibroblast growth factor (bFGF) (Tebu-Bio, Heerhugowaard, The Netherlands). Cells were exposed to laminar shear stress as previously described (Dekker et al., 2002), with the following modifications: cells

were seeded in a parallel plate type flow chamber (μ-slide I 0.6 luer (Ibidi, Martinsried, Germany)) coated with collagen IV and exposed to a calibrated mean shear stress level of 18 dyn/cm^2 for 5 days in EGM2 plus bFGF.

Lentiviral SOX18 transduction

The entire human SOX18 open reading frame (ORF) cDNA was cloned and expressed from the pRRL-cPPT-CMV-X2-PRE-SIN-IRES-eGFP vector (kindly provided by Dr. J. Seppen, Department of Experimental Hepatology, Academic Medical Center, Amsterdam, The Netherlands) as described previously (Fontijn et al., 2008). The resulting vector (further designated as SOX18) carried the CMV promoter followed by the SOX18 open reading frame, an internal ribosome entry site (IRES) and a green fluorescent protein (GFP) encoding sequence. As a control (further designated as mock), the same vector without SOX18 ORF was used. Lentiviruses were generated in HEK293T cells as described (Fontijn et al., 2008) using the CalPhos mammalian transfection kit (Clontech, Palo Alto, CA). ASCs were grown in EGM2 plus bFGF for 10 days. Subsequently, cells were replated and transduced at 80% confluency with SOX18- or mock virus in EGM2 plus bFGF medium for 24 h after which the cells were used for further experimentation.

Nuclear extraction and Western blot

Cytoplasmic and nuclear extracts of SOX18 transduced ASCs were prepared using the NucBuster protein extraction kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Protein content was determined using BCA reagent (Pierce, Rockford, IL, USA). Samples were denatured by boiling in sample buffer in the presence of 1% (w/v) SDS. Equal amounts of protein were separated on 10% SDS-PAGE followed by transfer to nitrocellulose for immuno-blot analysis. Blots were blocked in a 1:1 (v/v) dilution of Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and PBS. Incubations with primary and secondary antibodies were performed in a 1:1 (v/v) dilution of Odyssey blocking reagent and PBS containing 0.1% (v/v) Tween-20. After each incubation step, blots were washed 3 times for 5 min in PBS containing 0.1% (v/v) Tween-20. Primary antibody: anti-human SOX18 (Santa Cruz, CA, USA) dilution 1:200, incubation 4 °C overnight, secondary antibody: anti-rabbit IRDye 800 (LI-COR Biosciences) dilution 1:10,000, incubation 1 h at room temperature. Signals were recorded and quantified using an Odyssey infrared imaging system and Odyssey application software (LI-COR).

Immunofluorescence microscopy

ASCs were grown on collagen IV coated coverslips. Cells were washed with serum-free medium at 37 °C and fixed for 15 min with 4% paraformaldehyde. Phosphate buffered saline supplemented with 0.2% gelatin and 0.5% bovine serum albumin (BSA) was used as a blocking step and during incubations with primary and secondary antibodies. All incubations were performed for 1 h at room temperature. After each incubation step, coverslips were washed 4 times for 5 min with PBS. Antibodies were diluted according to the manufacturer's instructions. Primary antibody: anti-human SOX18 (Santa Cruz, CA, USA), secondary antibody: anti-rabbit Alexa Fluor 555 (Molecular Probes Eugene, OR, USA). Coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA) and images were recorded using a Leica DM6000 microscope and Leica Application Suite software (Leica Microsystems, Wetzlar, Germany).

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the Aurum total RNA mini kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions. 0.5 μg of total RNA was used for reverse transcription, with a (dT)12–18 primer using Superscript II (Invitrogen), according to the manufacturer's instructions. PCR reactions were performed in 10 μl volumes using Fast SYBR Green Master Mix (Applied Biosystems, Bleiswijk, The Netherlands) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Specificity of the amplification was checked by melt curve analysis. P0 levels were used for normalization. Primer sequences are given in Supplementary Table 1.

Flow cytometry

ASCs were detached by brief trypsinization followed by washing steps with medium and PBS containing 0.5% bovine serum albumin (BSA). Cells were incubated for 45 min with APC- or PE-labeled antibodies against KDR (VEGFR2) (Miltenyi Biotec, Leiden, The Netherlands) and CD34 (BioLegend San Diego, CA, USA). Cells were washed and surface expression was analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

In vitro angiogenesis model

3D human fibrin matrices were prepared by the addition of thrombin (0.05 U/ml) to a solution of 2 mg/ml fibrinogen (Kordia, Leiden, The Netherlands) in M199 medium. 100 μl was added to the wells of a 96-well plate. For network formation and subsequent polymerization, plates were incubated for one hour at room temperature followed by one hour at 37 °C. Thrombin was inactivated by the addition of medium; M199 (Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 10% human serum and 10% new born calf serum. Transduced ASCs were seeded at a density of 18.10^3 cells per well. After adherence of the cells overnight the medium was changed and, eventually, inhibitors were added. The experiments were terminated by washing in M199 and fixation with 2% paraformaldehyde for two hours. Tubular structures were recorded and quantified as described previously (Koolwijk et al., 1996). Matrices were embedded in paraffin and immunohistochemistry was performed as described previously (Naaijkens et al., 2010).

Chemotaxis

ASC chemotaxis was performed using fibronectin-coated 8 μm pore size Transwell inserts (Corning Inc. Life Sciences,

Lowell, MA, USA). ASCs were trypsinized, washed in PBS, resuspended in EGM2 without VEGF and seeded at a density of 15.10^3 cells per filter (0.33 cm^2). Cells were allowed to adhere for at least six hours after which the medium was changed to EBM2 (Lonza) supplemented with 0.5% HSA (Sanquin, Amsterdam, The Netherlands). VEGF was added to the lower compartment at a concentration of 25 ng/ml and cells were allowed to transmigrate for 16 h. Membranes were fixed in 4% PFA for 30 min and non-migrated cells were removed from the upper side of the membrane using cottonwool tips. Cells at the lower side of the filter were stained with Hoechst 33342. Transduced (GFP-positive) cells were quantified by counting 3 times 5 fields at 200× magnification using an inverted microscope.

Statistical analysis

Data are reported as mean and standard deviation (SD). Differences in mean values were analyzed using Student's *t*-test. Differences were considered significant at the $p < 0.05$ level.

Results

SOX18 shows nuclear localization upon transduction in ASCs

Early passage (p2–3) ASCs were grown on collagen IV matrices in EGM2 medium supplemented with bFGF, to provide conditions that were shown to enhance expression of endothelial features (Konno et al., 2010). We then sought to further improve this approach by combining these growth conditions with reprogramming of the cells by ectopic expression of the endothelial-specific transcription factor SOX18. SOX18 was transduced using a lentiviral vector containing the human SOX18 open reading frame (ORF) linked to an internal ribosome entry site (IRES) and a sequence encoding green fluorescent protein (GFP). Five days after transduction, monitoring of GFP expression revealed transduction efficiencies of >50%. Previously, we have shown cell type-specific expression of SOX18 in confluent endothelial cells, with a predominant nuclear localization of both endogenous and lentivirally-overexpressed SOX18 (Fontijn et al., 2008). Consistent with these earlier findings, SOX18 expressed in ASCs showed a major band of slightly less than 50 kDa on Western blot (Fig. 1a). Quantitative comparison of SOX18 protein in equal protein weight samples of cytoplasm and nucleoplasm revealed a 3.1-fold enrichment in the nucleus. This prevailing nuclear localization was confirmed by immunofluorescence microscopy (Fig. 1b).

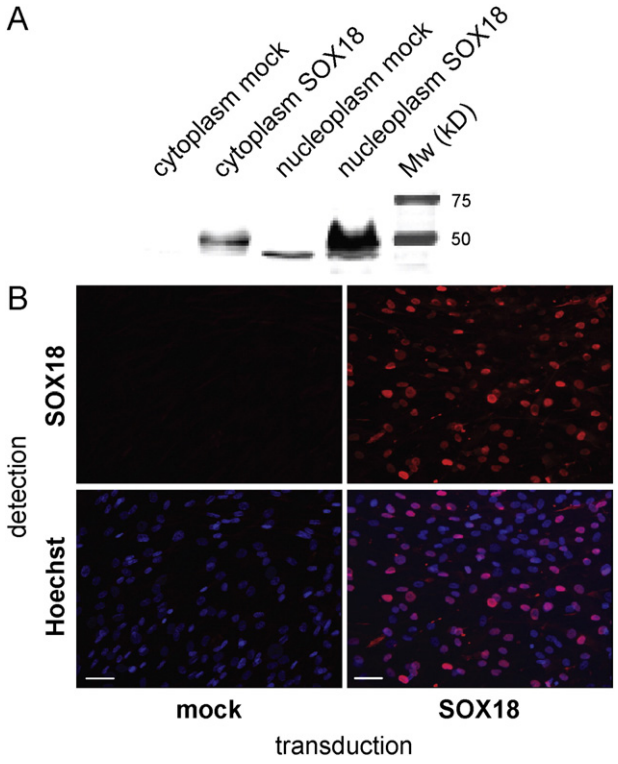

Figure 1 Nuclear localization of SOX18 in ASCs. ASCs were grown and transduced with mock or SOX18 lentivirus as described in Materials and methods. Five days after transduction, cells were analyzed for SOX18 subcellular localization by cell fractionation and immunofluorescence. (A) Cell fractionation. SOX18 distribution between cytoplasm and nucleoplasm was analyzed by SDS-PAGE and Western blotting. Lanes were loaded with 15 μg total protein. Detection was performed using antibodies raised against SOX18. (B) Immunofluorescent localization of SOX18. ASCs were grown on coverslips coated with collagen IV and transduced. After paraformaldehyde fixation, SOX18 localization was determined using an antibody raised against SOX18 (red). Nuclei were stained with Hoechst 33342 (blue). Bars: 25 μm.

SOX18 transduction in ASCs induces expression of (precursor) endothelial genes

After having established correct nuclear localization of SOX18 in heterologous ASCs, we determined the effects of SOX18 transduction on expression levels of a set of prototypical endothelial-specific genes. Cells were grown on collagen IV matrices in EGM2 medium supplemented with bFGF. Five days after SOX18 transduction, RNA was harvested from ASCs and mRNA levels of CDH5 (VE-Cadherin), PECAM1 (CD31), TEK (TIE2), vWF (von Willebrand factor), CD34, KDR (VEGFR2) and CLDN5 (claudin 5) were determined by semi-quantitative RT-PCR. vWF mRNA was not detected (data not shown). For CDH5, PECAM1, TIE2 and CLDN5, we observed a tendency towards an increase in expression, surpassing that of growth medium alone, upon transduction with SOX18 (Fig. 2a). However, with the exception of CLDN5, none of these genes reached significant levels of additional induction due to a considerable variation in response to SOX18 expression which is likely to reflect isolate- and transduction-dependent heterogeneity of the ASC cultures. Both basal- and SOX18-induced expression levels of all genes examined remained low as compared to primary endothelial cells (0.4% and less). Using immunofluorescence microscopy we observed expression of PECAM1 and VE-Cadherin in a limited number of SOX18-transduced cells whereas expression was not observed in mock-transduced controls. Both proteins displayed correct preferential targeting to the lateral membrane (Fig. 2b). In contrast to the aforementioned endothelial marker genes, KDR

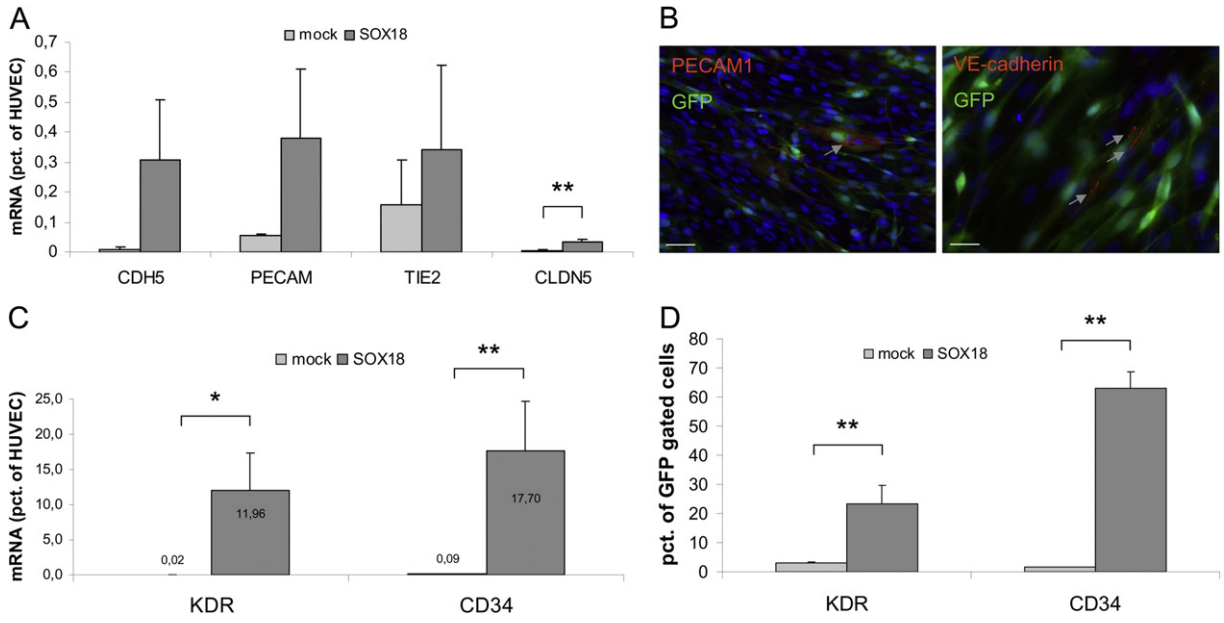

Figure 2 Effects of SOX18 transduction on expression of endothelial markers in ASCs. ASCs were grown and transduced with mock or SOX18 lentivirus as described in Materials and methods. Five days after transduction, cells were analyzed for expression of endothelial markers using semi-quantitative RT-PCR, immunofluorescence or flow cytometry. (A) mRNA levels of CHD5, PECAM, TIE2 and CLDN5 in SOX18- and mock-transduced ASCs relative to a pool of two isolates of human umbilical vein endothelial cells (HUVEC). Data are represented as mean and SD of 3 ASC isolates (**$p < 0.01$). (B) Immunofluorescent detection of PECAM and VE-Cadherin (both red). Transduced cells show GFP expression (green). Nuclei were stained with Hoechst 33342 (blue). Bars: 50 μm (PECAM) and 25 μm (VE-Cadherin). Arrows indicate PECAM1- or VE-Cadherin positive cell–cell contacts. (C) mRNA levels of KDR and CD34 in SOX18- and mock-transduced ASCs relative to HUVEC. Data are represented as mean and SD of at least 3 ASC isolates (*$p < 0.05$, **$p < 0.01$). (D) Flow cytometric quantification of KDR and CD34 expression in SOX18- and mock-transduced ASCs. GFP^+ cells were gated and the fraction of cells expressing KDR or CD34 was determined. Data are represented as mean and SD of 4 ASC isolates (**$p < 0.01$).

and CD34 showed significantly increased expression when ASCs were transduced with SOX18. The resulting expression levels approached levels measured in primary endothelial cells (Fig. 2c). FACS analyses confirmed KDR and CD34 positivity of GFP-gated populations (Fig. 2d).

SOX18 transduction in ASCs induces expression of morphogenic genes and affects chemotaxis

SOX18 target genes in endothelial cells included the matrix metalloprotease MMP7 and genes encoding molecules involved in vascular patterning (EFNB2), cellular guidance (SEMA3G) and chemoattraction (CXCR4) (Hoeth et al., 2012). Using semi-quantitative QPCR we observed that also in heterologous ASCs, SOX18 overexpression resulted in significantly increased levels of these genes (Figs. 3a, b). A major driving force in vascular morphogenesis is VEGF (Carmeliet et al., 1996), and we observed a marked induction of KDR (Figs. 2c, d). VEGF responsiveness would be a hallmark for the acquisition of endothelial functional properties by ASCs upon SOX18 transduction. We tested transmigration of SOX18 transduced ASCs through an 8 μM pore size fibronectin-coated Transwell membrane towards a VEGF gradient. Both SOX18 transduction and addition of VEGF tended to increase chemotaxis of mock-transduced cells but in both cases this did not reach significant levels (Fig. 3C). However, SOX18-transduced cells showed a significant 3.6-fold increase in VEGF-induced chemotaxis as compared to that in mock-transduced cells. These findings provide evidence that ectopic SOX18 expression in ASCs induces functional signaling in response to VEGF.

SOX18 transduction in ASCs induces morphologic changes, alters cell–cell and cell–matrix interactions and induces responsiveness to shear stress

When cultured under conditions that allow optimal proliferation, ASCs display an elongated, fibroblast-like spindle shape (Alt et al., 2011). In contrast, proliferating endothelial cells under static culture conditions tend to spread, flatten and, when grown to confluency, adopt a characteristic cobble-stone morphology. Here, we observed that growth of ASCs in EGM2 with bFGF medium aggravated the elongated cell shape as compared to growth in proliferation medium. Upon transduction with SOX18, ASCs showed spreading and flattening, resulting in a morphology reminiscent of endothelial cells (Fig. 4A). Shear forces are required for stabilization and patterning of blood vessels and, in adult life, maintain vascular homeostasis and drive arteriogenesis in collateral formation (Eitenmüller et al., 2006). During mouse embryonic development, SOX18 expression spatiotemporally coincides with the onset of circulation at 8 post coïtum (Pennisi et al., 2000a). Therefore, we tested the effects of SOX18 overexpression on shear-responsiveness of ASCs by exposing the cells to flow in a parallel plate chamber. We observed a striking

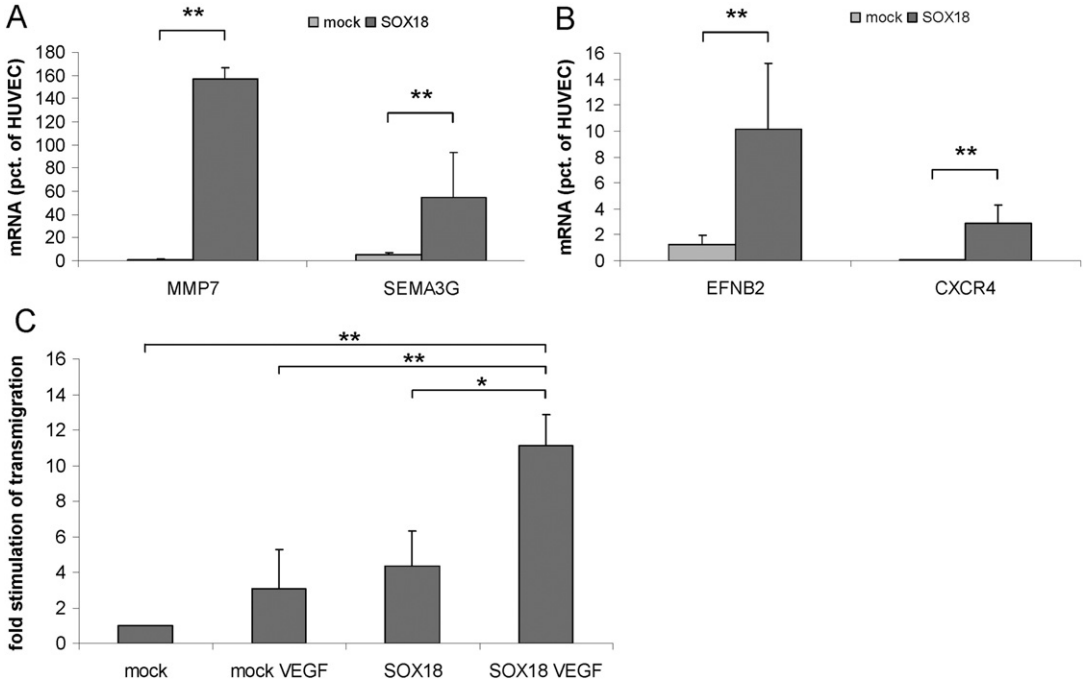

Figure 3 Effects of SOX18 transduction on expression of vascular patterning genes in ADCs and chemotaxis of ASCs. ASCs were grown and transduced with mock or SOX18 lentivirus as described in Materials and methods. Five days after transduction, cells were analyzed for expression of morphogenic genes and VEGF-induced chemotaxis. (A) Semi-quantitative RT-PCR for MMP7 and SEMA3G; mRNA levels in SOX18- and mock-transduced ASCs relative to HUVEC. (B) Semi-quantitative RT-PCR for EFNB2 and CXCR4; mRNA levels in SOX18- and mock-transduced ASCs relative to HUVEC. Data are represented as mean and SD of 5 ASC isolates. (**$p < 0.01$). (C) VEGF-driven chemotaxis measured as transmigration of SOX18- and mock-transduced ASCs across an 8 μm pore size Transwell filter coated with fibronectin. Cells were allowed to transmigrate against 25 ng/ml VEGF for 12 h after which filters were fixed and stained with Hoechst 33342. The number of spread GFP^+ cells at the bottom of the membrane was determined. Stimulation of transmigration was calculated relative to mock transduced cells. Results are represented as mean and SD of three isolates (*$p < 0.05$, **$p < 0.01$).

difference between mock- and SOX18-transduced cells with regard to their morphology and organization when exposed to prolonged laminar shear stress (5 days at an average of 18 dyn/cm^2). Whereas mock-transduced cells were dispersed throughout the matrix in a random orientation, SOX18-transduced cells were oriented parallel to the flow vector and formed elongated clusters (Fig. 4B), indicating that SOX18 expression contributes to plasticity of the cells by weakening cell–matrix interaction and enhancing cell–cell interaction. However, combination of overexpression of SOX18 with either shear stress or with overexpression of a major effector of shear stress in endothelial cells, the transcription factor KLF2 (Dekker et al., 2002, 2006; Boon & Horrevoets, 2009), had little effect on expression of genes described in the previous paragraphs, with the exception of TIE2 (Supplementary Fig. Fig. a). Only a partial shear responsiveness was thus observed, possibly due to differences in expression levels of components of the presumptive shear sensing complex, consisting of PECAM1, VE-Cadherin and KDR (Tzima et al., 2005) in SOX-transduced ASCs as compared to endothelial cells (Fig. 2a–d, Supplementary Fig. Fig. a). Integrins are known to be involved in endothelial shear responsiveness (Tzima et al., 2005). We observed that among the integrins tested, only integrin alphaV (ITGAV), known to be associated with angiogenesis and cell mobility, is increased upon overexpression of SOX18 (Fig. 4C). This finding is consistent with the observed VEGF-induced chemotaxis (Fig. 3C) and suggests increased plasticity of the SOX18-transduced ASCs under appropriate stimulatory conditions.

SOX18 transduced ASCs show spontaneous formation of tubular structures in 3D matrices

Next, we tested the behavior of SOX18 transduced ASCs in an *in vitro* model of sprouting tubes. SOX18 transduced ASCs were seeded and grown on three-dimensional matrices of fibrin as described in Materials and methods. 48 h after seeding, we observed spontaneous formation of branching tube-like structures in matrices seeded with SOX18-transduced ASCs while these structures were virtually absent in matrices seeded with mock-transduced cells (Fig. 5A). In contrast to what was previously observed in human microvascular endothelial cells (Koolwijk et al., 1996), the addition of combinations of TNF-α and bFGF or VEGF reduced rather than enhanced this tube formation (Supplementary Fig. Fig. b). Next, we performed morphological characterization of the tube-like structures. Differential interference contrast (DIC) microscopy was combined with detection of GFP that is co-expressed with SOX18 in transduced cells. Branched structures, consisting of multiple GFP-positive cells were found to enter the matrix up to a depth of approximately 250 μm (Fig. 5B). Using immunohistochemistry, expression of the SOX18 downstream markers CD34 and KDR was detected on sections of structures formed

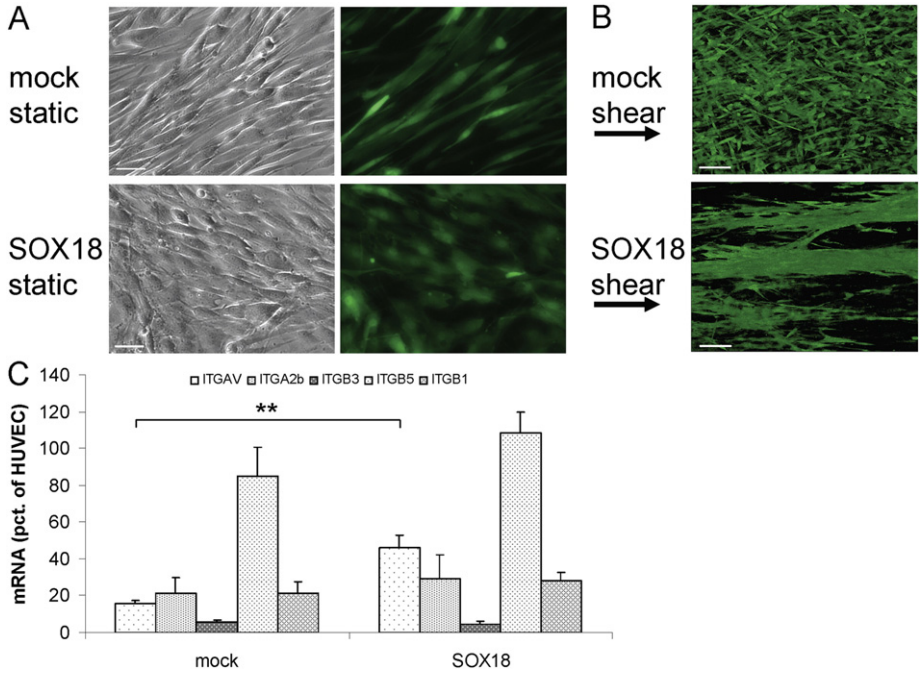

Figure 4 Effects of SOX18 transduction on ASC morphology, organization under shear stress and integrin subunit expression. ASCs were grown and transduced with mock or SOX18 lentivirus as described in Materials and methods. (A) Five days after transduction, cells were examined using phase contrast and immunofluorescence microscopy. Upper panel from left to right: mock transduced cells, static growth, phase contrast and corresponding GFP view. Lower panel from left to right: SOX18 transduced cells, static growth, phase contrast and corresponding GFP view. Bars: 25 μm. (B) After transduction, cells were seeded in a parallel plate-type flow chamber and exposed to five days of laminar shear stress at an average of 18 dyn cm^{-2}. The direction of laminar shear stress is indicated with an arrow. Shown is a projection of 20 confocal sections taken every 1.5 μm. Bars: 100 μm. (C) Five days after transduction, cells were analyzed for expression of mRNA encoding integrin (ITG) subunits using semi-quantitative RT-PCR. mRNA levels of ITGAV, ITGA2b, ITGB3, ITGB5 and ITGB1 in SOX18- and mock-transduced ASCs were determined relative to HUVEC. Data are represented as mean and SD of 3 ASC isolates (**$p < 0.01$).

specifically by SOX18-transduced ASCs (Fig. 5C). Both histochemistry and electron microscopy revealed extensive vacuolation associated specifically with structures formed in the matrix by SOX18 transduced cells, indicating active lumen formation being initiated by SOX18 (Fig. 5D).

SOX18-induced formation of tubular structures is dependent on MMP-7 activity and independent of VEGF

Since angiogenesis *in vivo* may occur in matrices of different compositions, we tested the ability of SOX18 transduced ASCs to form tubes in matrices composed of fibrin, collagen and mixtures of 10% collagen and 90% fibrin. In all cases SOX18 transduction enhanced formation of tubular structures by ASCs (Supplementary Fig. Fig. c). We then examined the contribution of two SOX18-induced genes, MMP7 and KDR, to the formation of tube-like structures by SOX18 transduced ASC. We quantified structures formed in the presence of the broad matrix metalloproteinase inhibitor Batimastat or in the presence of the VEGF inhibitor Avastin/Bevacizumab. As shown in Fig. 6A, inhibition of MMP activity significantly decreased formation of tubular structures whereas VEGF inhibition did not have a significant effect (Fig. 6B). Consistent with the latter observation, we found in an independent experiment that neither in the presence nor in the absence of VEGF, does Avastin or the KDR-kinase inhibitor SU1498 affect tube formation. The addition of an inhibitor of CXCR4 signaling, AMD3100/Plerixafor, did not affect tube formation either. Consistent with earlier findings in microvascular endothelial cells (Koolwijk et al., 1996), aprotinin/Trasylol significantly reduced tube formation, suggesting the involvement of plasminogen activation in tube formation by SOX18 transduced ASCs (Supplementary Fig. Fig. d). In view of the effect of Batimastat, we examined the possible induction of several MMPs in addition to the patterning MMP7. In contrast to MMP7 (Fig. 3A), none of the MMPs involved in endothelial sprouting: MMP1, MMP2, MMP3, MMP9, MMP12, and MMP14, showed significant induction of gene expression in response to SOX18 (Supplementary Fig. Fig. e). This makes MMP7 the most likely target for inhibition by Batimastat and suggests a crucial involvement of MMP7 in the process of tube formation by SOX18-transduced ASCs.

Discussion

Strategies aiming at controlled divergence of ASCs to specific differentiated cell types essentially rely on prolonged culture in the presence of specific sets of growth factors and/or matrix proteins (Zuk et al., 2001). Specific culture conditions were reported to induce subpopulations of ASCs to express

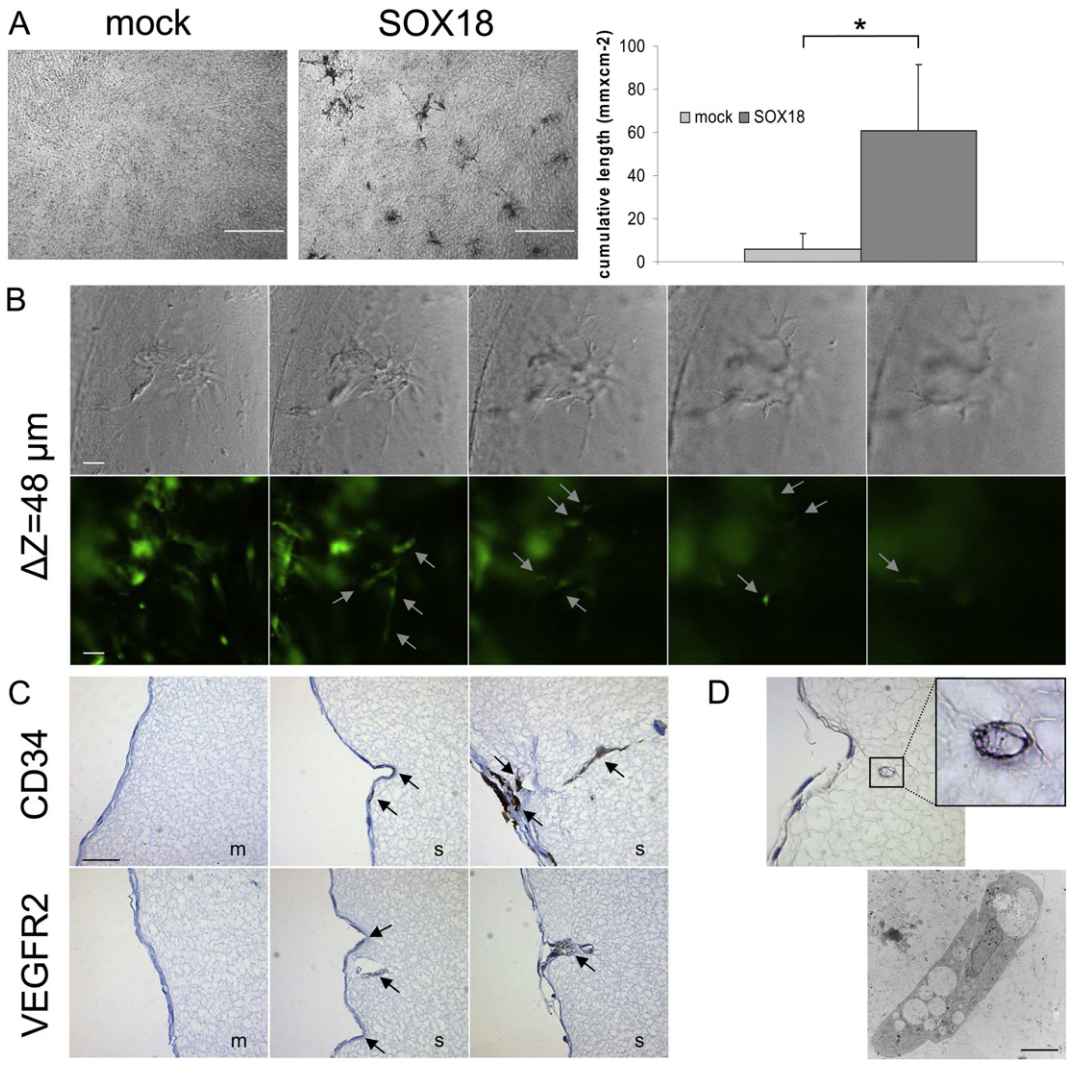

Figure 5 Tubular structures formed in 3D matrices by SOX18-transduced ASCs. ASCs were grown and transduced with mock or SOX18 lentivirus as described in Materials and methods. One day after transduction, cells were seeded on matrices and grown as described in Materials and methods. After 2 days the matrices were fixed with 2% paraformaldehyde. (A) Left two panels: tubular structures formed by mock and SOX18 transduced ASCs (phase contrast microscopy). Bars: 1000 μm. Right panel: total length of tubular structures was analyzed as described in Materials and methods. Data are represented as mean and SD of 3 ASC isolates (*$p < 0.05$). (B) Individual tubular structure, Z-intervals of 48 μm, upper row: differential interference contrast (DIC) microscopy, simultaneously, and in the same Z-plane, GFP signal of SOX18-transduced cells was recorded (lower row). Arrows indicate GFP fluorescence signal colocalizing with DIC signals. Bars: 50 μm. (C) After fixation, fibrin matrices were embedded in paraffin and sectioned at 5 μm thickness, perpendicular to the surface of the matrix, and stained for CD34 and VEGFR2 (brown/red). m: mock transduced ASCs, s: SOX18 transduced ASCs. Arrows indicate ingrowing cells that show positive staining. (D) Upper panel: hematoxilin stain, boxed individual vascular structure was recorded in more detail at 5-fold higher magnification (1000×). Lower panel: electron microscopy of SOX18 transduced ASCs in a fibrin matrix. Magnification 3400×, bar: 5 μm.

endothelial markers as PECAM1 (CD31) (Miranville et al., 2004), Tie2 (Konno et al., 2010), KDR and CD34 (Wosnitza et al., 2007; Konno et al., 2010). Reprogramming of fibroblasts to pluripotency, or direct conversion of fibroblasts to differentiated, functional cells, is based on transfer of specific sets of transcription factors (Takahashi & Yamanaka, 2006; Vierbuchen et al., 2010; Ieda et al., 2010). Human neonatal fibroblasts were transdifferentiated to endothelial cells, applying transduction of the induced pluripotent stem cell-factors Oct4 and Klf4 under inductive signaling conditions (Li et al., 2013). Here, we report on an approach that aims at controlled *in vitro* induction of endothelial features in ASCs by combining optimized culture conditions described by Konno et al. (2010) with direct reprogramming of the cells using SOX18, an endothelial-specific transcription factor that plays a key role in endothelial differentiation and development of the vasculature (François et al., 2010).

We could confirm that under the growth conditions applied, human ASCs show expression of the endothelial markers CDH5, PECAM1 and TIE2 at low levels (<0.4%) compared to primary endothelial cells. Additional transduction of ASCs with SOX18 tended to further increase expression of these genes, however,

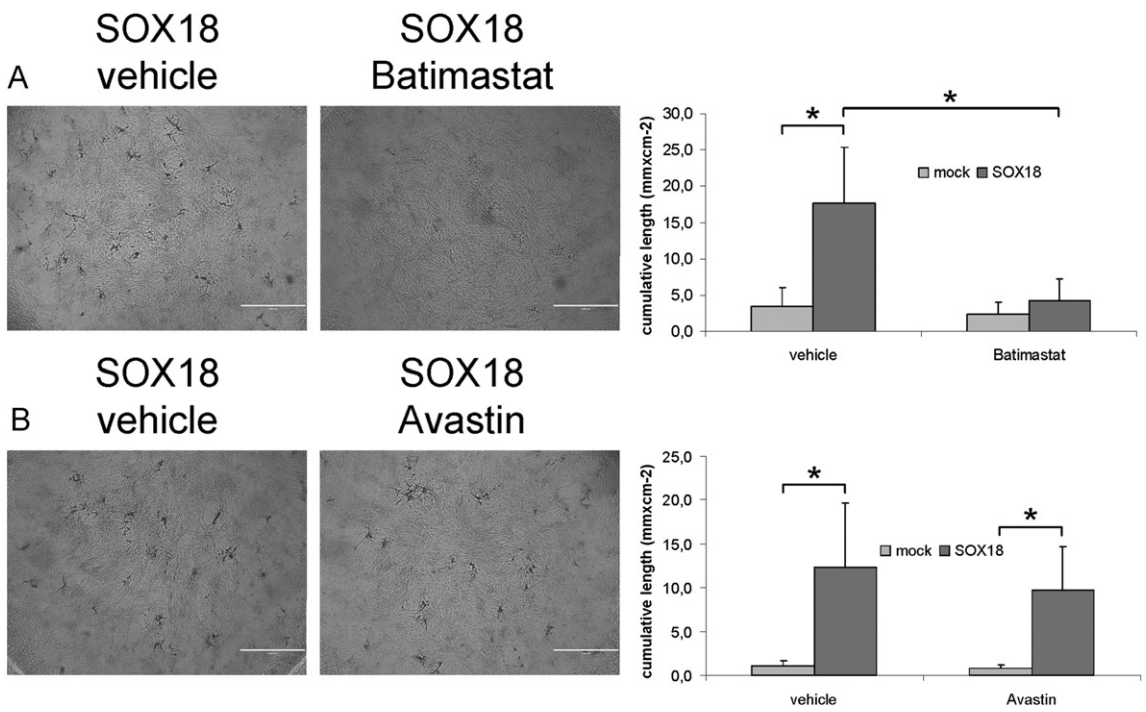

Figure 6 Effects of MMP- and VEGF-inhibition on formation of tubular structures in 3D matrices by SOX18-transduced ASCs. ASCs were grown and transduced with mock or SOX18 lentivirus as described in Materials and methods. One day after transduction, cells were seeded on preformed matrices and grown in the presence or absence of inhibitors as described in Materials and methods. After 3 days the clots were fixed with 2% paraformaldehyde. (A) MMP inhibition with 5 μg/ml (w/v) Batimastat. Left two panels: tubular structures were recorded using phase contrast microscopy. Bars: 1000 μm. Right panel: total length of tubular structures was quantified as described in Materials and methods. Data are represented as mean and SD of 3 ASC isolates (*$p < 0.05$). (B) VEGF inhibition with 200 μg/ml (w/v) Avastin. Left two panels: tubular structures were recorded using phase contrast microscopy. Bars: 1000 μm. Right panel: total length of tubular structures was quantified as described in Materials and methods. Data are represented as mean and SD of 3 ASC isolates (*$p < 0.05$).

this increase did not reach significant levels. CLDN5, a gene encoding the endothelial-specific tight junction protein claudin 5 and earlier described by our group as a direct target gene of SOX18 in HUVEC (Fontijn et al., 2008), showed significant upregulation upon SOX18 transduction in ASCs. Final mRNA levels amounted to a fraction of the levels observed in HUVEC and the protein could not be detected using immunofluorescence. In contrast, we found that SOX18 transduction specifically induced high expression levels of CD34 and KDR. Apart from their function in vasculogenesis and angiogenesis, KDR and CD34 are considered markers that occur in endothelial differentiation as early as the hemangioblast-stage. Their appearance, upon SOX18 transduction, together with the vascular patterning genes MMP7, EFNB2, SEMA3G and CXCR4, shows that in ASCs, ectopic expression of SOX18 is sufficient to induce a subset of genes involved in endothelial differentiation and vasculogenesis. The limited expression of markers of mature endothelial cells indicates that for further endothelial differentiation additional transcriptional programming, complementary to SOX18, is required. Here, it should be noted that in our experiments, SOX18 was constitutively expressed from a viral promoter, rather than from the native promoter which is likely subject to regulatory mechanisms.

In vivo, the function of SOX18 in endothelial differentiation and vascular development has proven difficult to dissect, due to the redundant function of members of the SOXF subfamily: SOX18, SOX17 and SOX7. Vascular anomalies observed in different animal models, including the naturally occurring *ragged* allelic mutants in mouse (Pennisi et al., 2000a; Downes et al., 2009), SOX18-null mouse (Pennisi et al., 2000b; François et al., 2008) and subsequent combined SoxF knock outs in mouse and zebra fish (Sakamoto et al., 2007; Herpers et al., 2008; Pendeville et al., 2008; Cermenati et al., 2008; Matsui et al., 2006; Sacilotto et al., 2013) point at the crucial roles of SOX18 in endothelial differentiation, arteriovenous specification, lymphangiogenesis and maintenance of vascular structural integrity. Young et al. (Young et al., 2006) studied the role of SOX18 in angiogenesis in an *in vitro* tube formation assay using endothelial cells. Overexpression of wild type SOX18 and the dominant negative SOX18 RaOp mutant resulted in gain and loss of tube formation, respectively. These studies reveal that correct spatiotemporal SOX18 expression is essential in several stages of vascular development.

In the experiments described in this report, molecular and cellular processes downstream of SOX18 were studied in a non-endothelial background. Effects of SOX18 expression on ASCs were manifested in an endothelial-like cell morphology and responsiveness to forces exerted by laminar flow. SOX18 induced responsiveness of ASCs to VEGF in a chemotaxis assay and rendered ASCs the capacity to enter 3D matrices of different compositions, migrate and form multicellular structures that reveal extensive intracellular

vacuolation, a process known to be an important component of luminogenesis in many vascular beds (Sacharidou et al., 2012).

Consistent with findings by Hoeth et al. in HUVEC (Hoeth et al., 2012), we observed that in ASCs, SOX18 modulates the expression of a repertoire of patterning genes, including MMP7. In addition, we found induction of KDR (VEGFR2), a morphogen receptor crucial in both vasculogenesis and angiogenesis (Carmeliet et al., 1996). Using the MMP inhibitor Batimastat, we could show that MMP activity is obligatory for formation of tubular structures and found that among the MMPs tested, only MMP7 showed significant upregulation upon SOX18 overexpression. Therefore, it seems likely that, in our model, MMP7 is essential for matrix degradation and thus a prerequisite for tube formation. MMP7 may efficiently degrade a broad range of extracellular matrix proteins, including fibrin and may regulate angiogenesis by activation, degradation and shedding of non-ECM proteins (Binni et al., 1999; Miyazaka et al., 1990; Ii et al., 2006). Inhibition of VEGF, using Avastin, or KDR kinase activity, using SU1498, did not affect tube formation, suggesting that the contribution of VEGF-dependent processes to tube formation is relatively small. Nevertheless, SOX18 transduced cells were capable of VEGF-dependent chemotaxis as shown in a transmigration assay. Interestingly, other guiding molecules, like SEMA3G and EFNB2 were induced, in addition to CD34 and CXCR4. Based on their function, these SOX18 target genes are plausible SOX18-effector molecules in our *in vitro* tube forming assay as well as in *in vivo* vasculogenic processes found to be directed by SOX18 in studies using reverse genetics (Sakamoto et al., 2007; Herpers et al., 2008; Pendeville et al., 2008; Cermenati et al., 2008; Matsui et al., 2006; Sacilotto et al., 2013). Sema3G was found to be associated with endothelial cells of the angiogenic arterial vasculature and showed binding to neuropilin receptors 1 and 2 (Kutschera et al., 2011). Mechanistically, the role of class 3 semaphorins in angiogenesis is poorly understood. Semaphorins may compete with VEGF for neuropilin binding and thus inhibit angiogenesis (Neufeld et al., 2012). In contrast, autocrine class 3 semaphorins may provide highly localized signals that antagonize endothelial integrin activation and thus enhance angiogenic plasticity (Serini et al., 2003). Ephrin-B2 is essential for proper vascular development during embryogenesis and was shown to be involved in arteriovenous specification (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998; Füller et al., 2003). Interestingly, in SOXF mutant zebra fish this arteriovenous specification is disturbed (Herpers et al., 2008; Pendeville et al., 2008; Cermenati et al., 2008). Furthermore, Ephrin-B2 contributes to sprouting angiogenesis and endothelial cell assembly by modulation of VEGR2 and VEGFR3 signaling (Sawamiphak et al., 2010; Wang et al., 2010). Taken together, the observed effects of SOX18 transduction on ASCs are consistent with findings in SOX18 animal models and point at controlled induction of some endothelial-like features that, at least in part, can be accounted for by induction of a specific set of SOX18-downstream genes.

Thus, our *in vitro* findings provide support for the hypothesis that ASCs acquire endothelial-like features upon reprogramming with SOX18. However, given the observed limited expression of endothelial-specific markers, additional factors will have to be defined to enhance ASC-endothelial transdifferentiation and proceed to *in vivo* studies. In conclusion, this *in vitro* study identifies SOX18 as a novel, promising factor for use in reprogramming strategies aimed at conversion of ASCs to endothelial cells for use in cardiovascular regenerative medicine.

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